# INVESTIGATIONS ON THE ANTIGENICITY OF SNAKE VENOMS

Final Technical Report

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## ABSTRACT

The chemical composition of viperotoxin, the neurotoxic protein isolated from the venom of Vipera palestinae has been determined. Viperotoxin is composed of one polypeptide chain cross-linked intramolecularly by three disulfide bridges. Lysine is in the amino-terminal position, and proline in the carboxy-terminal position of the viperotoxin chain.

Vipera palestinae hemorrhagin has been purified and isolated. The methods used involved chromatography on DEAE cellulose, Sephadex G-200 and DEAE-Sephadex A-50 pre-treated with large amounts of soybean trypsin inhibitor. The final product exhibited on immunodiffusion one precipitin line, and agar-gel electrophoresis showed a single protein band. Further tests have established that the hemorrhagin is an acidic protein with an estimated molecular weight of 44, 000. The purified hemorrhagin has gelatinase activity which can be inhibited by soybean trypsin inhibitor or DFP, while leaving the hemorrhagic activity intact. Among the possible explanations of this functional differentiation one may consider the association of two protein molecules thus far inseparable by chemical and physical methods, one molecule with two distinct active sites, or selective blocking of a part of one active center having two distinct biological activities.

Purified neurotoxin of V. palestinae (viperotoxin) was bound to tritiated alanine. This binding did not alter either the toxicity or the antigenicity of viperotoxin. In vivo distribution of sublethal doses of the labelled viperotoxin were followed in the various tissues of mice. The highest level of toxin was found in the kidneys, followed by liver, lungs and spleen, and a surprisingly low concentration in the brain. The clearance rate from the blood was slow.

# REPORT

The studies carried out during the period covered by this Report were in the following main areas:

(1) Determination of the chemical composition of the neurotoxin (viperotoxin) of Vipera palestinae venom

(2) Purification and final isolation of Vipera palestinae hemorrhagin

(3) Distribution and localization of Vipera palestinae neurotoxin in the animal body

(4) The effect of neurotoxin-associated lipids on the toxicity of Vipera palestinae venom

Parts (1) and (2) have been completed during the year of these studies; Part (3) has been well advanced, whereas part (4) has only begun and is still far from being completed. Attempts to concentrate and purify antivenins have also been initiated but are still in the developmental stage.

## (1) Chemical composition of viperotoxin

In the previous Annual Report for the 2nd year of these studies, a complete account was given on the isolation and chemical physical characterization of the neurotoxin (viperotoxin) of Vipera palestinae. In the present Report, details of the chemical composition of viperotoxin are presented[1].

The neurotoxic preparation remaining in solution after precipitation with trichloroacetic acid (final concentration, 3.3%) was tested for its lipid content by extraction with methanol-chloroform. The lipid fraction did not contain phospholipids, as indicated by a comparison with lecithin, lysolecithin and serum phospholipids on thin-layer chromatography. The chromatograms were developed in chloroform-methanol-water (65:25:4, v/v) and detected with rhodamine B. In contrast to the phospholipids mentioned, the lipid extracted from the purified neurotoxic preparation moved with the

solvent front under the conditions of chromatography. No protein-bound sugar was detected in the viperotoxin either before or after the removal of lipids.

The amino acid composition of viperotoxin is given in Table 1. The amino acid content was determined, after acid hydrolysis, in a Beckman-Spinco automatic amino acid analyzer. Cystine was determined also by quantitation of S-carboxymethylcysteine found in hydrolyzates of fully reduced and carboxymethylated viperotoxin (5.8 residues of S-carboxymethylcysteine per molecule of viperotoxin, assuming a mol. wt. of 11.500). Tryptophan content of viperotoxin was obtained by spectro-photometric analysis. The approximate number of the different amino acids in viperotoxin was calculated assuming 10 residues of aspartic acid per molecule. This fitted the assignment of one residue of methionine per viperotoxin molecule, and gave a calculated minimal mol. wt. of 12212, in good agreement with determinations of the molecular weight in the ultracentrifuge.

No free sulfhydryl groups were found in viperotoxin either upon titration with dinitrobenzoyl disulfide or upon amino acid analysis of hydrolysates of viperotoxin treated with iodoacetic acid. On the other hand, total reduction of viperotoxin with $\beta$-mercaptoethanol in 8 M guanidine-HCl followed by reaction with iodoacetic acid yielded a derivative containing 6 S-carboxymethylcysteine residues per molecule and sedimenting in the ultracentrifuge similarly (1.5 S at a concentration of 0.2%) to native viperotoxin. It may be concluded, therefore, that viperotoxin is composed of a single polypeptide chain, cross-linked by 3 disulfide bonds, and devoid of cysteine sulfhydryl groups.

The reduced and carboxymethylated viperotoxin derivative possesses no toxic activity. On the other hand, exposure to iodoacetic acid did not impair the original toxicity of native viperotoxin.

Viperotoxin has only one amino-terminal amino acid, lysine, as detected by the dinitrophenylation technique. As bis-DNP-lysine is not well separated from bis-DNF-tyrosine by two-dimensional thin-layer chromatography according to Randenra...[2], the eluted DNP-amino acid was esterified and the resulting DNP-amino acid methyl ester was chromatographed in parallel with bis-DNP-tyrosine methyl ester and bis-DNP-lysine methyl ester, according to Burstein and Patchornik[3].

Efforts to split off the carboxy-terminal amino acid of viperotoxin with DFP-treated carboxypeptidase A were unsuccessful under a variety of conditions. No free amino acids were detected either on paper chromatography or in the amino acid analyzer, after treatment of both native viperotoxin and of fully reduced and carboxymethylated viperotoxin with carboxypeptidase for as long as 24 h. Experiments performed in the presence of 8 M urea led to similar negative results. On the other hand, after hydrazinolysis of viperotoxin according to Akabori et al[4], free proline was detected on amino acid analysis.

The presence of carboxy-terminal proline in viperotoxin was confirmed by the method of Wilchek et al.[5]. These authors have shown that sodium in liquid ammonia cleaves in a polypeptide chain selectively peptide bonds involving the amino group of proline, and that this technique may be used for the determination of carboxy-terminal proline. Thus, a yield of 93% free proline was obtained on application of this method to ovalbumin. When viperotoxin was reacted with sodium in liquid ammonia according to Wilchek et al., free proline was obtained in a yield of 85% of the expected value, assuming a mol wt. of 12000.

(2) Purification and final isolation of Vipera palestinae hemorrhagin

The hemorrhagic fraction obtained by chromatography on DEAE-cellulose was concentrated by lyophilization to 1.5% and further fractionated by precipitation with saturated $(NH_4)_2SO_4$ solution (54% at $pH_7$). The pre-

cipitate was washed twice with 60% $(NH_4)_2SO_4$ solution. dissolved in distilled water and dialyzed against 0.9% NaCl solution (300 vol.). The dissolved precipitate contained, in addition to the hemorrhagin, capillary permeability-increasing activity, proteases active on gelatin but not on casein, and phospholipase A. The dissolved precipitate was chromatographed on Sephadex G-200 and two protein fractions were obtained, the first possessing hemorrhagic and proteolytic activities and the second phospholipase A. The capillary permeability-increasing action was confined to the second fraction. The peak hemorrhagic activity was not located at the peak proteolytic activity. The Sephadex G-200 hemorrhagic fraction represented 20% of the total protein eluted from the column. At peak hemorrhagic activity the lethal dose was 3.5 ug per mouse, as compared to 15 ug per mouse for the DEAE-cellulose hemorrhagic fraction. Immunodiffusion of the pooled "Sephadex-hemorrhagic fraction exhibited 3 precipitin lines.

Chromatography of the pooled Sephadex G-200 hemorrhagic fraction was carried out on DEAE-Sephadex A-50 column which had previously been treated with large amounts of soybean trypsin inhibitor. Two protein fractions were obtained after applying ionic strength gradient. The first contained hemorrhagic and proteolytic activities, the second having a high protein content due to elution of soybean trypsin inhibitor and still containing proteolytic but no hemorrhagic activity. The recovery of protein in the hemorrhagic fraction eluted from the soybean trypsin inhibitor-(STI)-treated DEAE-Sephadex column was 20%. Its lethal activity was 8.0 ug per mouse. Rechromatography on a DEAE-Sephadex column furnished a single symmetrical peak. On immunodiffusion it exhibited one precipitin line. Agar-gel electrophoresis of this fraction showed one protein band near the application line. On immunoelectrophoresis a single precipitin line corresponding to the protein band in the agar-gel electrophoresis was seen. In the ultracentrifuge this protein sedimented as a single boundary. From the $S_{20,w}=3.44$ S, the diffusion coefficient $D_{20,w}=7.6.1o^{-7}$ $cm^2/sec$, and a partial specific volume of 0.75, an apparent mol. wt. of 44000 was estimated.

As seen in Table 2, the proteolytic activity of the ammonium sulphate precipitated hemorrhagic fraction was completely inhibited by STI (5% final concentration), whereas the hemorrhagic activity remained unaffected. Smaller amounts of STI (0.5% and 1.0%) had no inhibitory effect. The effect of di-isoprobyl fluorophosphate (DFP) ($5.10^{-3}$M) was similar to that of STI. E-amino caproicacid even in high amounts (5% final concentration) inhibited neither the hemorrhagic nor the proteolytic activities. On the other hand, treatment with EDTA ($5.10^{-3}$M) inactivated both the proteolytic and the hemorrhagic activities.

Capillary permeability increasing activity and phospholipase A were note inhibited by DFP, STI or EACA at the above concentrations. Treatment with EDTA ($5.10^{-3}$M), however, abolished completely phospholipase A activity, leaving the capillary permeability increasing activity unaffected.

Both STI and DFP completely inhibited the proteolytic activity of the purified hemorrhagin, eluted from the STI-treated DEAE-Sephadex column, whilst leaving its hemorrhagic action unaffected.

(3) Distribution and localization of Vipera palestinae neurotoxin (viperotoxin) in the animal body

The neurotoxin of Vipera palestinae venom was isolated and its chemical characteristics determined. The mechanisms by which viperotoxin exerts its neurotoxic activity and its specific site of action are not yet understood. The knowledge of its distribution and localization in the envenomated animal may contribute to a better understanding of viperotoxin mechanism of action.

The purified viperotoxin was labelled with tritium by means of binding tritiated DL-alanine to the $\varepsilon$-amino groups of lysine in the viperotoxin molecule. Amino acid analysis of unlabelled alanilated neurotoxin indicated that the binding ration was about 7 molecules of alanine per molecule of viperotoxin. The binding – which was carried out under mild conditions –

was found not to affect significantly the toxicity and the antigenic structure of viperotoxin as determined by mouse inoculation and immunodiffusion tests.

The labelled viperotoxin was separated completely from unbound radioactive material and was found to possess a specific activity of 28 mc/mg protein. Sub-lethal doses of the labelled viperotoxin were injected into mice and the in vivo distribution was followed by radioassay of aliquots of tissues.

10 minutes after the injection, the highest level (26% of the injected dose) was found in the kidneys followed in a decreasing order by liver, lungs and spleen, with the lowest in the brain; 40% of the injected dose were still present in the blood, indicating a clearance rate slower than that of a compared protein possessing similar physico-chemical properties (ribonuclease). Moreover, the percentage of the injected dose of viperotoxin was about 3 times higher in the liver, spleen and lungs than that of the compared protein, this may be due to a stronger affinity of viperotoxin to tissues. Comparison of the concentration in the brain relatively to its concentration in blood revealed that it was 1:126 of the value calculated for kidneys.

1 hour after viperotoxin injection, the distribution pattern was similar, except for lower levels in the tissues, and the mentioned differences between viperotoxin in the compared protein still persist. The percentage of the concentration in the blood which was found in the brain was 1:270 of that found in the kidneys. These results show that viperotoxin hardly penetrates through the blood-brain barrier and its concentration in the brain does not increase with time. The concentration in the brain is apparently too small to account for the neurotoxic impairment.

Similar experiments carried out with $I^{131}$-labelled viperotoxin revealed that radioiodination cannot be used for in vivo tests since deiodination occurs.

(4) The effect of neurotoxin-associated lipids on the toxicity of Vipera palestinae venom

In previous studies[1], the purification of Vipera palestinae neurotoxin was achieved by chromatographic and precipitation methods. The purified neurotoxin was found to be much less toxic than the crude venom. The extraction of the lipid from the neurotoxin caused a 7 fold decrease in its toxicity. It was tried to restore the toxicity by recombination of the neurotoxin and the lipid. Various quantities of the extracted lipid were added to the purified neurotoxin and the mixture was incubated at 37°C for 30'. A partial restoration of the toxicity was achieved; the $LD_{50}$ of the mixture was found to be 180 ug as compared to 250 ug for the purified neurotoxin. Variation in the micellar state of the added lipid caused an additional increase in toxicity - 1 $LD_{50}$ = 160 ug. The final product was 4.3 times less toxic than a 3.3% TCA-soluble neurotoxin preparation, compared to which the purified neurotoxin was 7 times less toxic. Increasing the ratio of the lipid to neurotoxin up to 18 fold ratio in the crude venom and in the TCA- soluble neurotoxin, or recombination at low temperature proved to be inefficient in raising the toxicity.

Table 1

Amino Acid Composition of Viperotoxin

The results given are the average recoveries of two analyses after hydrolysis of 0.6 mg of viperotoxin for 22 h.

| Amino acid | umoles in sample | Ratio to aspartic acid | Number of residues per molecule* | Mol. wt. |
|---|---|---|---|---|
| Lysine | 0.4747 | 8.86 | 9 | 1153.5 |
| Histidine | 0.1716 | 3.20 | 3 | 411.4 |
| Arginine | 0.3293 | 6.20 | 6 | 937.0 |
| Aspartic acid | 0.5355 | 10 | 10 | 1150.8 |
| Threonine | 0.1765 | 3.30 | 4 | 404.4 |
| Serine | 0.3051 | 5.70 | 6 | 522.4 |
| Glutamic acid | 0.5160 | 9.64 | 10 | 1291.1 |
| Proline | 0.6477 | 12.12 | 12 | 1165.3 |
| Glycine | 0.5370 | 10.02 | 10 | 570.5 |
| Alanine | 0.3174 | 5.94 | 6 | 427.0 |
| Half cystine | 0.3096 | 5.80 | 6 | 612.8 |
| Valine | 0.1890 | 3.52 | 4 | 396.5 |
| Methionine | 0.0465 | 0.88 | 1 | 131.2 |
| Isoleucine | 0.2020 | 3.78 | 4 | 452.6 |
| Leucine | 0.1980 | 3.70 | 4 | 452.6 |
| Tyrosine | 0.1900 | 3.54 | 4 | 652.7 |
| Phenylalanine | 0.2550 | 4.76 | 5 | 735.9 |
| Truptophan** | | | 4 | 744.8 |
| Total | | | 108 | 12212.6 |

* Assuming 10 residues of aspartic acid per molecule; values were rounded to the closest integral number.

** Tryptophan was determined by spectrophotometric analysis.

Table 2

Effect of Inhibitors on Enzymatic and Toxic Activities of the Ammonium Sulphate Precipitated Fraction

Final concentrations in assay systems: DFP $5.10^{-3}$M, STI 5%, EACA 5%, EDTA $5.10^{-2}$M, venom fraction 0.6 mg/ml. The incubated mixtures were titrated with parallel controls devoid of inhibitor. Assay for hemorrhagic activity by i.v. injection of 1-8 $LL_{50}$.

| Activities* | DFP | STI | EACA | EDTA |
|---|---|---|---|---|
| Hemorrhagin | + | + | + | - |
| Protease | - | - | + | - |
| Capillary permeability increasing fac tor | + | + | + | + |
| Phospholipase A | + | + | + | - |

* + active, - inactive (100% inhibition)

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